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# Sexual dimorphism in water and nitrogen use strategies in *Honckenya peploides*: timing matters

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## Abstract

### Aims

Sexes of dimorphic species often differ in ecophysiological traits and display spatial segregation. These differences have been interpreted as an evolved response of the sexes to meet the specific resource demands associated with reproduction. Sexes may differ not only in the amount of resources allocated to reproduction but also in the timing of allocation to reproduction. In this study, we hypothesize that as a consequence of their specific resource demands for reproduction, the sexes of the dune plant *Honckenya peploides* differ in terms of temporal patterns of water use efficiency and nitrogen use and acquisition.

### Methods

Water use efficiency, as inferred from leaf carbon isotope discrimination ( $\Delta^{13}\text{C}$ ), nitrogen use, estimated by leaf nitrogen isotope composition ( $\delta^{15}\text{N}$ ), and the foliar carbon and nitrogen contents were measured in males and females at three different points in time.

### Important Findings

Females had greater water use efficiency than males, regardless of time. The ratio of  $\text{N}^{15}$  to  $\text{N}^{14}$  did not change with time in males, but

significantly decreased in August for females. The total N content in the leaf tissues of females decreased as the season progressed, while in males a decrease was only found from April to June and then it remained constant from June to August. A similar pattern, but reversed, was followed by the foliar C/N ratio. Additionally, negative relationships between leaf  $\Delta^{13}\text{C}$  and N content were found at all times for males and only at the end of the season for females. Thus, our hypothesis that sex-specific patterns of nitrogen and water use efficiency will depend on time was supported. Overall, our results highlight the importance of including time in studies of sexual dimorphism, and also the role that physiological specialization plays in meeting the specific demands associated with reproduction.

**Keywords:** cost of reproduction, *Honckenya peploides*, leaf nitrogen content, sexual dimorphism, stable isotopes, water use efficiency

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## INTRODUCTION

Sexual dimorphism is common in many dioecious plants, with males and females usually differing not only in their sexual characters but also in other aspects such as growth, morphology and physiology (Dawson and Bliss 1989; Dawson and Ehleringer 1993; Geber *et al.* 1999 and references therein). These intersexual differences in secondary traits have been explained as the result of females and males having different reproductive roles (dispersal of seeds vs pollen) that impose different demands for

resources (Delph 1990; Sánchez Vilas *et al.* 2011). For example, females usually allocate more resources, particularly in terms of carbon, to reproduction than males because they produce fruits in addition to flowers (Antos and Allen 1994; Jonasson *et al.* 1997; Obeso 2002). Accordingly, females tend to invest proportionally more of their resources in reproduction and less in other functions, such as maintenance and growth compared to males (Barrett and Hough 2013; Delph 1990; Obeso 2002).

Females and males may differ not only in the absolute amount of resources allocated to various functions but also in the timing

of allocation to vegetative growth and reproduction. In most dioecious species, males tend to flower earlier than females; however, females have to bear the cost of seed provisioning later in the season (Barrett and Hough 2013; Obeso 2002). If males and females incur their maximum reproductive effort at different times, differences in how they manage the resources available are also expected. The different sexes may adjust the proportion of resources allocated to vegetative biomass with time, so that sexual dimorphism depends on the life stage considered (Sánchez Vilas and Pannell 2011; Teitel *et al.* 2016). Males and females may also adopt different physiological strategies for resource acquisition and use to satisfy their different temporal reproductive demands. Since pollen is usually rich in nitrogen, while seeds and fruits mainly contain starch and lipids (Wallace and Rundel 1979), males would be selected for greater nitrogen uptake, at least during flowering, while females would be selected for greater carbon gain (Dawson and Geber 1999). The study of plant physiology in species with two or more distinct reproductive individuals is rapidly increasing (Correia and Díaz Barradas 2000; Culley *et al.* 2006; Juvany and Munné-Bosch 2015; Morales *et al.* 2013; Sánchez Vilas and Retuerto 2009, 2011; Zunzunegui *et al.* 2005), including studies that use long-term indicators of physiological performance (Álvarez-Cansino *et al.* 2012; Montesinos *et al.* 2012; Verdú 2004; Yang *et al.* 2014). Long-term indicators, such as leaf isotope composition, reflect average physiological activity of the leaf over its lifetime and therefore are more precise than instantaneous observations. However, the temporal dimension, i.e., comparisons between the sexes at different life stages—crucial to differentiate sex-specific dynamics—is rarely considered in these studies.

Carbon isotope composition ( $\delta^{13}\text{C}$ ), commonly expressed as a discrimination ( $\Delta^{13}\text{C}$ ) against a source (i.e., atmospheric  $\text{CO}_2$ ), provides information about processes such as leaf conductance, hydraulic capacity, potential water use efficiency and photosynthetic capacity (Casper *et al.* 2005; Dawson *et al.* 2002; Ehleringer 1993; Farquhar *et al.* 1989; Maguas and Griffiths 2003), which, in contrast to gas exchange, is integrated over time (from the moment the leaf tissue is synthesized).  $\delta^{13}\text{C}$  (or  $\Delta^{13}\text{C}$ ) has also been used to compare water use strategies between males and females of dioecious species (Dawson and Bliss 1989; Dawson and Ehleringer 1993; Retuerto *et al.* 2000). Because  $\text{CO}_2$  and  $\text{H}_2\text{O}$  share the same diffusion pathway through stomata, absolute increases in carbon gain is likely to result in greater water loss and consequently in reduced water use efficiency. If indeed females have been selected to maximize carbon gain, we would expect that they may be less water use efficient than males. However, this expectation may not hold if females are also under stronger selection to improve resource use efficiency (Geber *et al.* 1999). Evidence for the latter has been identified in previous research, with females showing traits that favour water conservation, such as smaller leaves or more allocation to roots than males (Sánchez Vilas and Retuerto 2009; Sánchez Vilas *et al.* 2012).

$\delta^{15}\text{N}$  has been used less extensively in plant ecophysiology, but it has been proposed as an integrative trait to assess

stress tolerance in plants (Lopes *et al.* 2004; Robinson 2000) and to study integrated plant N acquisition, assimilation and use (Filella and Peñuelas 2003). However, the interpretation of  $\delta^{15}\text{N}$  is more complex than that of  $\delta^{13}\text{C}$ , and predictions are not straightforward because multiple processes and factors determine plant  $\delta^{15}\text{N}$  (Evans 2001; Handley *et al.* 1994; Hogberg 1997). Plant  $\delta^{15}\text{N}$  will be determined not only by the source of N (different forms of N available to plants, such as  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , have different isotopic composition) but also by physiological mechanisms within the plant; different uptake mechanisms (e.g. direct uptake of soil N vs uptake mediated by symbiotic microorganism), assimilation pathways (e.g. in roots or shoots) and recycling of N in the plant can influence  $\delta^{15}\text{N}$  (Evans 2001).

Here, we examine how males and females of the coastal dune plant *Honckenya peploides* differ in the nutrient content of their vegetative tissues (nitrogen and carbon/nitrogen ratio), as well as in physiological traits, particularly in their integrated water use efficiency and nitrogen use and acquisition strategies, at different times throughout the season. Specifically, we compared the temporal patterns of these traits in males and females by measuring carbon and nitrogen contents and isotope composition in leaf samples harvested over time. Because sexes may differ not only in the amount of resources allocated to reproduction but also in the timing of allocation to reproduction (Ehlers and Thompson 2004; Hemborg and Karlsson 1999), we hypothesize that sex-specific patterns of nitrogen and water use efficiency will depend on time. Finally, in this study, we also investigate the relationships between  $\Delta^{13}\text{C}$  and foliar nitrogen content, for which the literature does not show a consistent trend (Damesin *et al.* 1997; Farquhar *et al.* 1989; Retuerto *et al.* 2000; Sparks and Ehleringer 1997) and extended the ecological information on the relationships between  $\Delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

## MATERIALS AND METHODS

### Study species

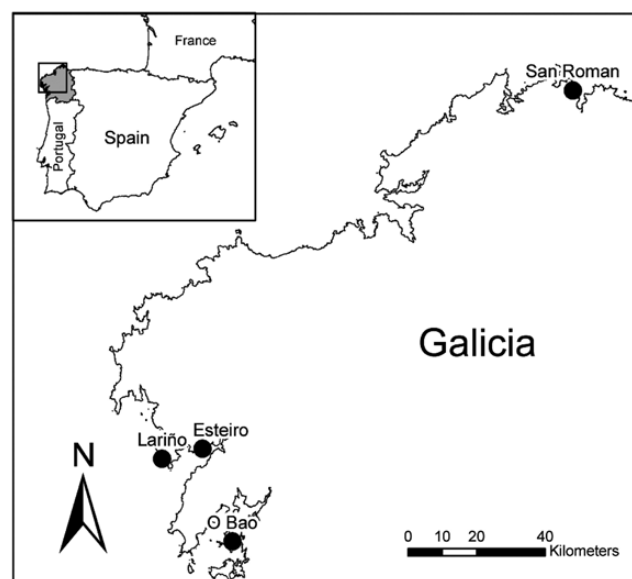
Sea sandwort, *H. peploides* (L.) Ehrh. (Caryophyllaceae), is a perennial plant species with a sexual system consisting of two distinguishable morphs: one that never produces pollen and is constant in their sex expression (i.e. females) and another that produces pollen but may also produce a small number of seeds. We will refer to this second sexual morph as male since it attains most of its fitness via pollen production and export. This system, called subdioecy, is close to dioecy in the evolutionary pathway from hermaphroditism to dioecy via gynodioecy (for a review, see Delph and Wolf 2005). *H. peploides* has a circumpolar distribution (from temperate to Arctic zones; also anthropochorous in South America). The range of the *H. peploides* on the Iberian Peninsula extends from the Atlantic coast of northern Portugal and northwards and eastwards along the Bay of Biscay to France. It is a hemi-cryptophytic plant, which regrows every spring from long rhizomes that produce compact groups of aerial shoots and which



forms vegetative patches or mats. These patches are typically found on the upper beach, at the foot of the foredune, and contribute to form small mounds called embryo dunes. This species is an early colonizer, which thus contributes to stabilization and anchorage of the soil and facilitates the establishment of other species (Gagné and Houle 2001; Houle 1997). It is fairly common in sandy beaches throughout its distribution area, and in the Northwest of Spain, our study region, *H. peploides* displays an extreme spatial segregation of the sexes, with monomorphic patches composed exclusively of individuals of either one sex or the other (Sánchez Vilas *et al.* 2010). The plants reproduce sexually by seed or clonally by rhizomes. Flowers are axillary and solitary and/or in one- to six-flowered terminal cymes, and are strongly honey scented. Two types of flower can be found in *H. peploides*: one type ('pistillate') has long styles, short petals and non-functional anthers, while the other ('staminate') has short styles, long petals and long stamens that produce pollen grains (Tsukui and Sugawara 1992). The latter type rarely produces seeds, and when it does the number of seeds is very low compared to female flowers (fruit sets in natural populations range from 0 to 2.2% for staminate flowers and from 12% to 76.5% in pistillate flowers; Tsukui and Sugawara 1992). Both types of flower have nectaries, which attract pollinators, at the base of the stamens. In accordance with Tsukui and Sugawara (1992) and following the descriptions of Lloyd (1976) and Delph (1990), we will hereafter refer to plants with pistillate flowers as females and to plants with staminate flowers as males. The sex determination system in *H. peploides* is considered heterogamous for the males, as their seeds develop into female and male plants in an approximate 1:3 ratio. Seeds of female flowers produce about as many females as males (Malling 1957). No evidence of environmental sex determination has been found to date.

### Sampling sites

Sampling was carried out in April, June and August 2005, at four sites on the coast of Galicia (Fig. 1): San Román (7°37'35"W, 43°43'17"N), Lariño (9°6'41"W, 42°45'51"N), Esteiro (8°58'3"W, 42°47'25"N) and O Bao (8°51'42"W, 42°32'49"N). The climatic characteristics (mean annual precipitation and temperature) of the meteorological stations close to each of the populations can be seen in Fig. 2. We conducted the study on segregated, clearly recognizable, unisexual patches of plants. At each site, we studied two patches, one composed by females and the other by males. At some sites (O Bao and San Román), no more patches were available on the same beach, so patch replication was not possible. The size of the male and female patches sampled (in square metres) was 21.4 and 71.2 (Lariño), 95.5 and 37.9 (O Bao), 224.0 and 35.8 (San Román) and 129.5 and 20.5 (Esteiro). The distance between male and female patches at each site (in metres) was 650 (Lariño), 625 (O Bao), 82 (San Román) and 85 (Esteiro). Individuals in the patches were sexed on the basis of their floral morphology, which was consistent



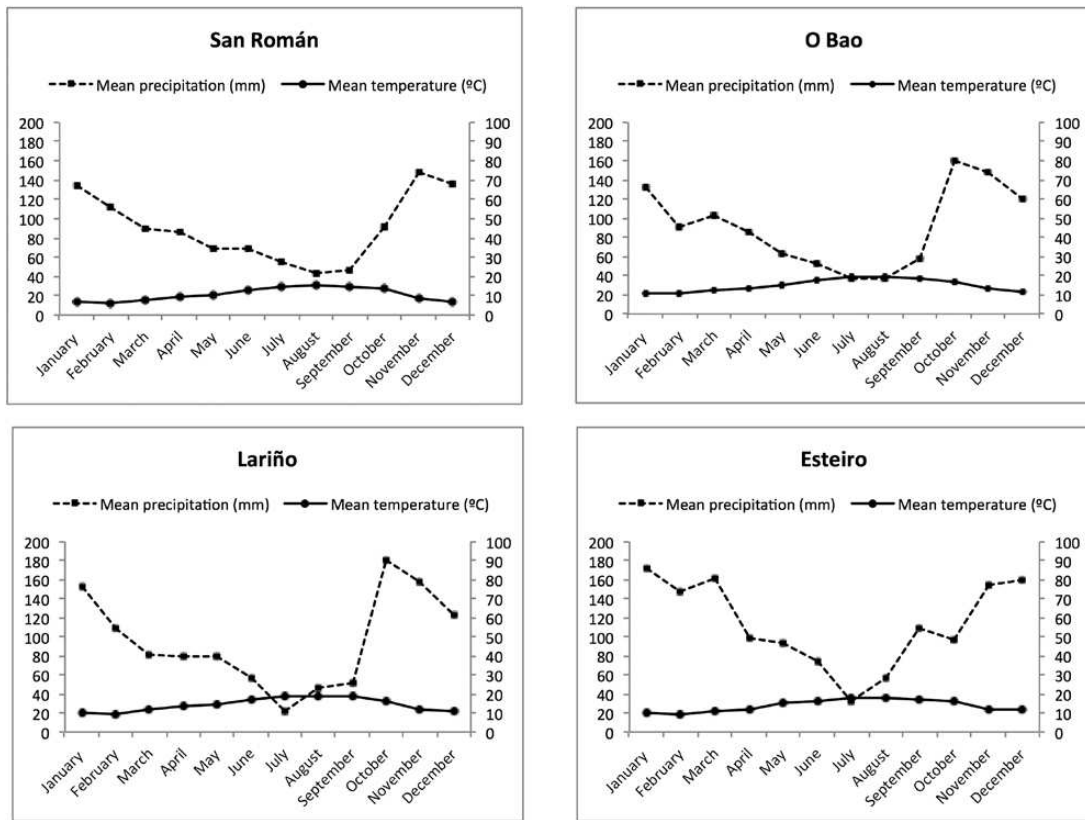
**Figure 1:** geographical location of sampling sites.

with the two previous years (J. Sanchez Vilas & R. Retuerto, personal observation). In April, we randomly sampled 14 plants bearing reproductive structures and 14 vegetative plants (flower and fruits absent) from each patch (28 plants in a male patch and 28 plants in a female patch at each site, 224 samples in total). In June and August, most of the individuals were reproductive, and therefore only 14 reproductive individuals per patch were sampled (14 plants in a male patch and 14 plants in a female patch at each site; 112 samples in total). To avoid resampling of the same ramets (clonal individuals), the minimum distance between two samples was 50 cm. Sánchez Vilas *et al.* (2010), using amplified fragment length polymorphism and isozyme analysis, found considerably high values of genetic variation within each of these unisexual patches; based on the spatial distribution of that variation, a distance between samples >50 cm should be enough to ensure that different genets were sampled.

### Soil characteristics

Soil samples (0–35 cm) were collected using a cylindrical soil core sampler (10-cm diameter, 45-cm length) from male and female patches at Lariño, O Bao and San Román in July 2005. The total number of samples collected for each sex at each site can be found in [supplementary Table S1](#). No soil samples were taken in Esteiro, as plants were growing on pebbles. Samples were sealed in air-tight plastic bags and transported to the laboratory where they were weighed and subsequently dried at 45°C for 6 days. Dry weight was then measured, and water content (g water Kg<sup>-1</sup> dry soil) was calculated as follows: (wet soil – dry soil) × 1000/dry soil.

Total C and N contents (%) were determined in a subsample of soil (~2 mg) that was ground in a pestle and mortar to a fine powder (<2 mm) for analysis with an elemental analyser (CHNS-932; LECO Corp., St Joseph, MI, USA).



**Figure 2:** Gaussen's ombrothermic diagrams for the meteorological stations nearest to each of the sampling sites (Penedo do Galo, for San Román; Lira, for Lariño; Muros, for Esteiro; Corrubedo, for O Bao). Dashed and solid lines represent, respectively, monthly average precipitation (millimetre) and temperature (degree Celsius) in a 2:1 (temperature:precipitation) scale. Drought conditions are considered when the precipitation is lower than twice the temperature values. Data are means for at least 5 years.

Soil salinity was determined by electrical conductivity in a 1:2.5 soil:water suspension, with an EC meter (524 Crison, Crison Instruments, Barcelona, Spain).

### C, N and isotope analyses

From each sampled plant, we collected the four most apical, fully expanded leaves that were then cleaned of organic debris, dried at 50°C for over 5 days and finely ground using an ultracentrifugal mill (Retsch ZM200). The concentrations of total C and N and the molar  $^{15}\text{N}/^{14}\text{N}$  and  $^{13}\text{C}/^{12}\text{C}$  ratios were determined on subsamples (ca. 1 mg dry wt) using an elemental analyser (FlashEA 1112 Series) coupled to an isotope ratio mass spectrometer (ThermoFinnigan MAT253, Bremen, Germany). Leaf isotope ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) were expressed relative to the composition of a standard (Pee Dee Belemnite [PDB] calcium carbonate for C and air for N).  $\delta$  Values (‰) were calculated as  $\delta = ([R_{\text{sam}}/R_{\text{std}}] - 1) \times 1000$ , where  $R$  refers to the  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$  ratio in the plant sample ( $R_{\text{sam}}$ ) and standard ( $R_{\text{std}}$ ), respectively. As a system check of analysis, polyethylene (IAEA-C6) and  $(\text{NH}_4)_2\text{SO}_4$  (IAEA N1) were used as international isotope secondary standards for C and N, respectively. Values of  $\delta^{13}\text{C}$  were converted into carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) by use of the following expression:  $\Delta^{13}\text{C} = (\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{plant}}) / (1 + [\delta^{13}\text{C}_{\text{plant}} / 1000])$

(Farquhar *et al.* 1989) and assuming a  $\delta^{13}\text{C}$  air value of  $-8.0\text{‰}$  on the PDB scale.  $\Delta^{13}\text{C}$  provides a long-term integrated measure of many ecophysiological processes, including photosynthesis and transpiration (Farquhar *et al.* 1989) and has been mainly used as a surrogate for potential water use efficiency (Ehleringer 1993). In C3 plants,  $\Delta^{13}\text{C}$  values generally lie between  $\sim 15\text{‰}$  and  $25\text{‰}$  for plants with high and low water use efficiencies, respectively (Dawson *et al.* 2002).

### Data analysis

All statistical analyses were conducted using R v. 2.8.1 (R Development Core Team 2008). Differences in soil characteristics between male and female patches were tested by means of Wilcoxon rank sum tests, by means of the 'wilcox.test' function in R. Variation in  $\Delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and foliar nutrient content, as expressed by the C/N ratio and the foliar N content, was evaluated using linear mixed-effects models, using the 'lme' function from the R-package 'nlme' (Pinheiro *et al.* 2015). Sex (male and female) and time (April, June and August) were fitted as fixed factors, and patch nested within locality was fitted as a random factor. For the first sampling date, April, the effect of the reproductive status on  $\Delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and foliar nutrient content was analysed by linear mixed-effects models with sex and reproductive status (reproductive

and non-reproductive) as fixed factors and patch nested within locality as random factor. The inclusion of the random-effects nested factor was made in order to account for any unmeasured variation across patches (at each locality, males and females were growing in separate patches) and thus make the hypothesis testing for sex of primary interest. Significance of the fixed effects and their interaction was assessed by means of likelihood ratio tests that compared models with and without the term of interest, fitted using maximum likelihood estimates (Crawley 2007; Pinheiro and Bates 2000). Main effects were not tested when included in a significant interaction.

Standardized major axis (SMA) regression was used to estimate the significance of the relationships between  $\Delta^{13}\text{C}$  and foliar N content and also to compare the slopes and the elevations (intercept) of the linear relationships between the sexes at each sampling date. In particular, common slope was tested by means of the likelihood ratio statistic and then compared to a chi-square distribution. If the fitted lines for each sex shared a common slope, the elevations were then compared using Wald tests (Warton *et al.* 2012). These analyses were carried out using the package ‘smatr’ in R (Warton *et al.* 2012).  $\Delta^{13}\text{C}$  and foliar N content were log-transformed prior to the SMA analysis, and the assumptions of normality, linearity and homocedasticity of the residuals were evaluated visually through graphs (Warton *et al.* 2006).

## RESULTS

### Soil characteristics

Patches of male and female plants occurred on soil with similar characteristics, as shown by the lack of significant differences in most variables measured (supplementary Table S1). Differences were only found in O Bao, where female plants occurred on soil with a higher water content and total carbon in their soil than male plants (supplementary Table S1).

### Carbon and nitrogen isotope signatures

At the beginning of the season, carbon isotope discrimination was affected by reproductive status of the plants (Rep; Table 1), regardless of the sex (Sex, Sex  $\times$  Rep; Table 1), with reproductive individuals having higher values (i.e. lower

water use efficiency; Fig. 3A). However, overall (averaging the different times throughout the season), females had significantly lower carbon isotope discrimination (i.e. higher water use efficiency) than males (Sex; Table 2). Carbon isotope discrimination also changed significantly throughout the season (Time; Table 2) and followed a similar trend for both males and females (Time  $\times$  Sex; Table 2), with the lowest values in April and the highest values in June (Fig. 4A).

The nitrogen isotope composition of the plants did not differ between reproductive and non-reproductive individuals (Table 1; Fig. 3B) or between the sexes at the beginning of the season (Table 1). However, the temporal patterns of nitrogen isotope composition were significantly different for males and females (Sex  $\times$  Time; Table 2); in particular, the  $^{15}\text{N}/^{14}\text{N}$  ratio decreased as the growing season progressed in females but not in males (Fig. 4B).

### Carbon to nitrogen ratio and foliar nitrogen content

The foliar N content and the C/N ratio were significantly affected by the reproductive status of the plants (Rep; Table 1), regardless of their sex (Sex, Sex  $\times$  Rep; Table 1), with reproductive plants having lower foliar N content and greater C/N ratios than non-reproductive plants (Fig. 3C and D).

The foliar N content and the C/N ratio changed significantly with time, and the pattern of change was significantly different for males and females (Sex  $\times$  Time; Table 2). Foliar N content decreased from April to June in both sexes, while further decrease from June to August occurred in females but not in males (Fig. 4C). C/N ratio increased from April to June in both sexes, while further increase from June to August was only significant for females (Fig. 4D). Overall, females experienced a greater reduction in their foliar N content and a greater increase in their C/N ratio than males from the start to the end of the season (Fig. 4C and D).

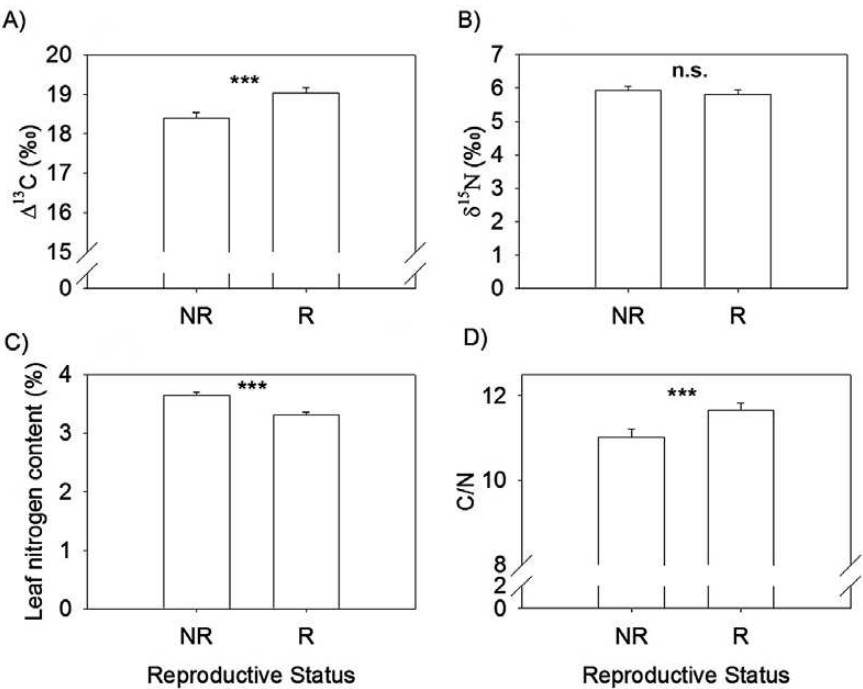
### Relationship between carbon isotope discrimination and foliar N content

At the beginning of the season (April), there was a significant negative relationship between  $\Delta^{13}\text{C}$  and foliar N content in both non-reproductive and reproductive males, but not in females (Figs. 5A and B). Similarly, in June, males but not females showed a significant negative relationship between

**Table 1:** results of the linear mixed-effects model for leaf carbon isotope discrimination ( $\Delta^{13}\text{C}$ ), leaf nitrogen isotope composition ( $\delta^{15}\text{N}$ ), leaf N content (%DW) and leaf carbon to nitrogen ratio (C:N) for male and female plants of *Honckenya peploides* (i.e. Sex) examined at different reproductive status (i.e. Rep, Vegetative and Reproductive) in April

Source of variation	$\Delta^{13}\text{C}$			$\delta^{15}\text{N}$			N (%DW)			C/N		
	L ratio	df	P	L ratio	df	P	L ratio	df	P	L ratio	df	P
Sex	1.19	1	0.276	0.0776	1	0.78	1.13	1	0.288	1.08	1	0.298
Rep	27.7	1	<b>&lt;0.001</b>	1.05	1	0.306	30	1	<b>&lt;0.001</b>	15.8	1	<b>&lt;0.001</b>
Sex $\times$ Rep	$3.36 \times 10^{-3}$	1	0.954	0.131	1	0.717	0.623	1	0.43	1.12	1	0.29

Values in bold are significant at  $P < 0.05$ . Likelihood ratio tests were carried out for all variables removed from the model one at a time.



**Figure 3:** mean values ( $N = 112 \pm 1$  SE for (A) leaf carbon isotope discrimination,  $\Delta^{13}\text{C}$ , (B) leaf nitrogen isotope composition,  $\delta^{15}\text{N}$ , (C) foliar nitrogen content and (D) leaf carbon to nitrogen ratio in reproductive (R) and non-reproductive (NR) individuals of *Honckenya peploides*. The asterisks indicate significant differences: \*\*\* $P < 0.001$ ; n.s. = non-significant.

**Table 2:** results of the linear mixed-effects model for leaf carbon isotope discrimination ( $\Delta^{13}\text{C}$ ), nitrogen isotope composition ( $\delta^{15}\text{N}$ ), leaf N content (%DW) and leaf carbon to nitrogen ratio (C:N) for male and female plants of *Honckenya peploides* examined at different times throughout the season (Time: April, June, August)

Source of variation	$\Delta^{13}\text{C}$			$\delta^{15}\text{N}$			N (%DW)			C/N		
	L ratio	df	P	L ratio	df	P	L ratio	df	P	L ratio	df	P
Sex	5.52	1	<b>0.018</b>	—	—	—	—	—	—	—	—	—
Time	95.17	2	<b>&lt;0.001</b>	—	—	—	—	—	—	—	—	—
Sex $\times$ Time	0.279	2	0.879	14.76	2	<b>&lt;0.001</b>	26.81	2	<b>&lt;0.001</b>	22.24	2	<b>&lt;0.001</b>

Values in bold are significant at  $P < 0.05$ . Likelihood ratio tests were carried out for all variables removed from the model one at a time; main effects included in a significant interaction were not tested (—).

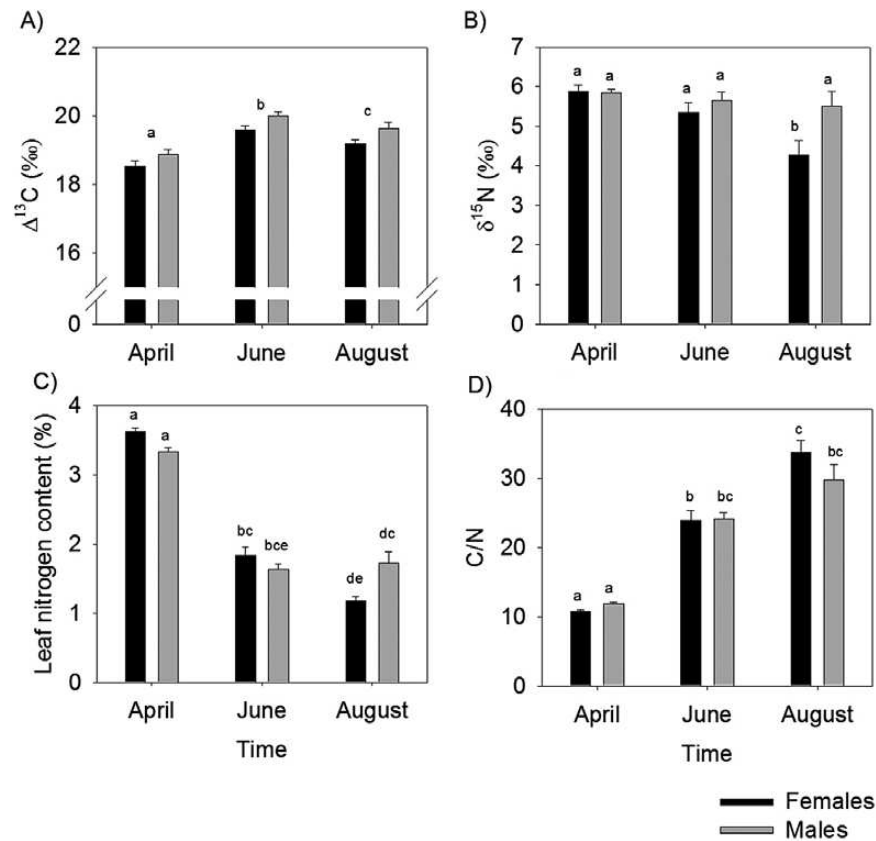
$\Delta^{13}\text{C}$  and foliar N content (Fig. 5C). However, in August, there was a significant relationship between  $\Delta^{13}\text{C}$  and foliar N for both males and females, and they also shared a common slope (Fig. 5D; males: estimate [95% confidence interval {CI}] =  $-0.116 [-0.145, -0.092]$ , females:  $-0.143 [-0.184, -0.110]$ ;  $L$ -ratio statistic = 1.44, degrees of freedom [df] = 1,  $P = 0.230$ ). Taking into account the common slope, we compare the elevation (intercept) of the SMA fitted lines between males and females for August and we did find a significant difference (males: estimate [95% CI] =  $1.31 [1.30, 1.32]$ , females:  $1.29 [1.28, 1.29]$ ; Wald statistic = 19.22, df = 1,  $P < 0.001$ ).

DISCUSSION

Our results showed differences in physiological traits between males and females of *H. peploides*. In contrast to our expectations,

we found that females were more efficient in the use of water than males—as indicated by the carbon isotope discrimination data,  $\Delta^{13}\text{C}$ , with females having lower values than males (Fig. 3A; Table 1)—suggesting that they consume less water per unit carbon fixed. Sex-specific differences in carbon isotope discrimination have been found in several dioecious species, although the differences do not follow a general pattern and males have been found to have either lower (Dawson and Ehleringer 1993; Marshall *et al.* 1993; Ward *et al.* 2002), higher (Dawson and Bliss 1989; Jones *et al.* 1999) or similar values than females (Kohorn *et al.* 1994; Leigh and Nicotra 2003). Very few carbon isotope discrimination studies have been carried out in other sexually dimorphic, non-dioecious species (but see Case and Barrett 2001; Verdú 2004). In *H. peploides*, females allocated greater biomass to reproduction than males (Sánchez Vilas and Retuerto 2012), as they need to





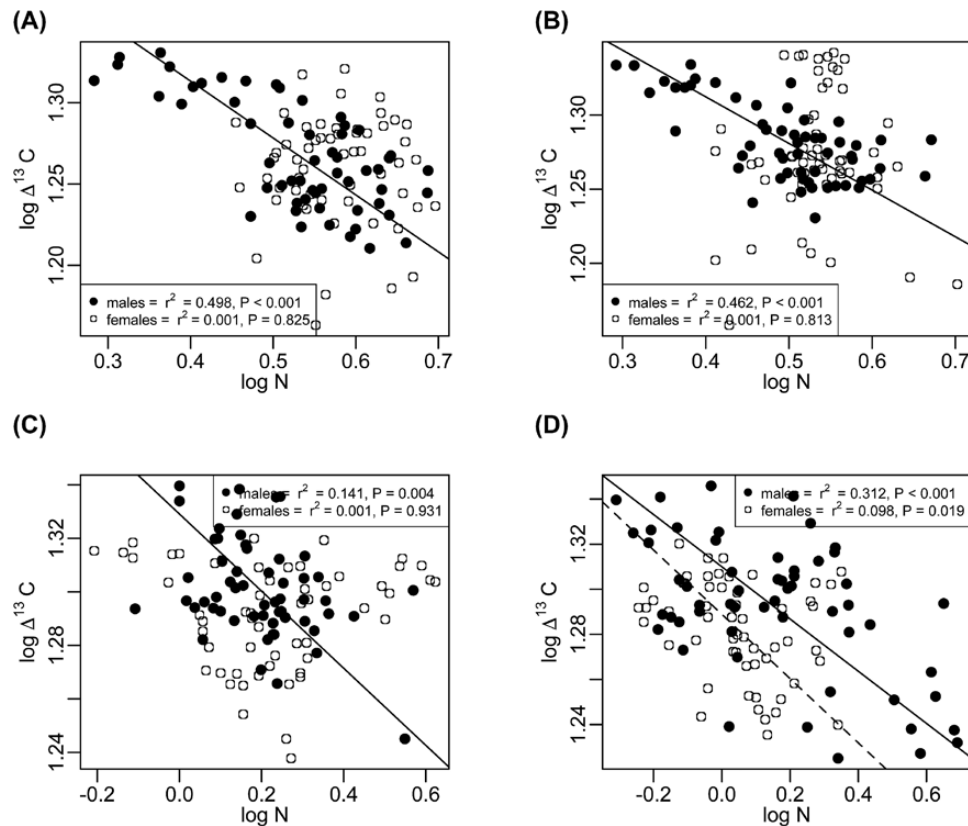
**Figure 4:** temporal variation in (A) leaf carbon isotope discrimination,  $\Delta^{13}\text{C}$ , (B) leaf nitrogen isotope composition,  $\delta^{15}\text{N}$ , (C) leaf nitrogen content and (D) carbon to nitrogen ratio in leaf tissues of male and female plants of *Honckenya peploides*. Bars represent means ( $\pm 1$  SE).  $N = 112$  for April, and  $N = 56$  for June and August. Different letters above bars indicate statistically significant differences. Note that for (A) letters indicate differences in time (regardless of sex), as no significant interaction sex  $\times$  time was found (see Table 2 for more details).

support developing fruits as well as flowers. In order to meet the higher carbon demands associated with developing fruits, females may have increased carbon assimilation rates, which may lead to a greater water loss and consequently a reduced water use efficiency compared to males. The role of reproduction affecting water use efficiency is indeed consistent with the finding that all reproductive plants at the beginning of the season had reduced water use efficiency in comparison to non-reproductive plants. However, greater carbon demands associated to female reproduction did not explain our results on carbon isotope discrimination. This, at first, appears to be a surprising result, particularly because females of *H. peploides* seem to occupy either similar or moister sites than males (this study and Sánchez Vilas *et al.* 2012), and therefore, we could expect females to be less conservative in the use of water than males. However, as suggested by Sánchez Vilas and Retuerto (2011), developing fruits in *H. peploides* are green and thus may significantly contribute to carbon requirements reducing the relative cost of fruit production, as seen in other species (Aschan and Pfan 2003; Yu *et al.* 2013) and therefore potentially explaining a lack of increased carbon assimilation at leaf level. In addition, water diverted to fruits (in addition to flowers) may cause greater water loss in females than in

males, which ultimately may explain both their more conservative water use and their preference for moister sites. This is also consistent with previous findings showing that females of *H. peploides* have mechanisms to maintain more favourable water content in their tissues than males, such as smaller leaves, lower specific leaf areas and higher biomass allocation to roots (Sánchez Vilas and Retuerto 2009; Sánchez Vilas *et al.* 2012).

Because of its biochemical properties, the Rubisco enzyme discriminates against  $^{13}\text{CO}_2$  more than it does against the much more abundant  $^{12}\text{CO}_2$  (Farquhar *et al.* 1989). Thus, carbon isotope discrimination is greatly dependent on the ratio of intercellular ( $C_i$ ) to atmospheric ( $C_a$ )  $\text{CO}_2$  concentrations (Farquhar *et al.* 1982, 1989). Increased carboxylation efficiency (higher photosynthetic rates; Ehleringer 1994) and/or diffusional restrictions to  $\text{CO}_2$  uptake by reductions in stomatal conductance may cause a decrease in  $C_i$  at the active site of Rubisco and consequently a lower discrimination against the heavy carbon isotope. Therefore, increased photosynthetic capacity could be—in this way—explaining a lower discrimination against  $^{13}\text{C}$  in females. However, reduced stomatal conductance in females is perhaps a more plausible explanation and would be in accordance with both





**Figure 5:** relationships between leaf carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) and foliar nitrogen content (%DW) of male and female plants of *Honckenya peploides* at different reproductive stages (A: non-reproductive, B: reproductive) and at three dates (A and B: April, C: June and D: August). Lines are only shown for significant relationships (solid lines = males, dashed lines = females).

a more conservative use of water by females and also with our results on the relationship between leaf  $\Delta^{13}\text{C}$  and N content. Negative relationships between leaf  $\Delta^{13}\text{C}$  and N content may occur as consequence of the relationship between the photosynthetic capacity of leaves and their N content (Evans 1989): high concentrations of N can increase photosynthetic rates, drawing down intercellular  $\text{CO}_2$  concentrations and reducing Rubisco discrimination against  $^{13}\text{C}$ . The fact that a significant negative relationship between leaf  $\Delta^{13}\text{C}$  and N content was found consistently for males all throughout the season, but only in August for females, indicates that factors others than leaf N content play a major role in regulating the intercellular  $\text{CO}_2$  concentrations in females. For example, differences in stomatal conductances between males and females could help to explain the observed pattern; if females have lower stomatal conductances, this can lead to lower intercellular  $\text{CO}_2$  irrespective of leaf N content. Lower stomatal conductances in females than males have been found for other dioecious species (e.g. Correia and Díaz Barradas 2000; Crawford and Balfour 1983) and as suggested above, would be in accordance with our results on leaf  $\Delta^{13}\text{C}$  (greater efficiency in the use of water in females than in males). The role of the environmental context cannot be neglected, particularly as the significance of the relationship for females depended on time,

and previous studies have found that both light and water availability can influence the relationship between leaf  $\Delta^{13}\text{C}$  and leaf N content (Guehl *et al.* 1995; Retuerto *et al.* 2000; Roiloa *et al.* 2014; Sparks and Ehleringer 1997). Here, we only measured water content towards the end of the season and found that females grew either at sites with similar water contents or in wetter soil than males (see also Sánchez Vilas and Retuerto 2012).

The increased carbon discrimination (decreased water use efficiency) observed from spring (April) to summer (June and August) was similar for both sexes, and it was probably more the result of a reduction in photosynthetic rates at the end of the growing season than the consequence of increased stomatal conductances. That is, photosynthetic constraints prevailed over stomatal conductance in determining  $C_i$ . The temporal variation in leaf nitrogen content (Fig. 4C) supports this interpretation (see further discussion below). The lower leaf nitrogen content in summer (especially for females in August) relative to spring should result in a decreased carboxylation efficiency, high  $C_i$  and consequently increased carbon discrimination. Increased  $\Delta^{13}\text{C}$  in water-stressed plants has been related to a significant reduction in leaf nitrogen content (Guehl *et al.* 1995; Retuerto *et al.* 2000; Roiloa *et al.* 2014; Sparks and Ehleringer 1997).

The ratio of  $N^{15}$  to  $N^{14}$  in the leaf tissues decreased in August only for females, with male having a similar leaf nitrogen composition all throughout the season (Fig. 4B). These differences in the patterns of leaf  $\delta^{15}N$  between male and females may reflect differences in a range of processes and factors, such as the  $\delta^{15}N$  of the nitrogen source, intra-plant fractionation events that occur during assimilation processes, nitrogen losses from the plant and/or even the presence of mycorrhizal associations. Due to the complexity of the underlying mechanisms that can influence variation in leaf nitrogen isotope composition (Filella and Peñuelas 2003; Robinson 2000), we are unable to determine the cause of the intersexual differences. High leaf  $\delta^{15}N$  early in spring could indicate nitrogen losses in the soil after N cycle processes (denitrification, ammonia volatilization and nitrate leaching, all contribute to  $\delta^{15}N$  enrichment of the remaining nitrogen) are activated by spring rains and increasing temperatures (Filella and Peñuelas 2003). However, increasing  $\delta^{15}N$  values could also be expected as the season progresses and water becomes scarce, limiting the uptake and further transfer of N to the upper plant parts (Lopes and Araus 2006). It is therefore difficult to explain the observed patterns in terms of water availability alone and the interplay between soil N availability and intra-plant N partitioning may also play an important role. Several studies support a positive association between foliar  $\delta^{15}N$  and soil N availability (see Garten *et al.* 2011 and references therein). However, we failed to detect differences in the total nitrogen content—measured towards the end of the season—between the soil of males and females. It is worth to note that conventional measures of soil N availability do not necessarily correspond to plant-available N (Franklin *et al.* 2009), and the isotopic signature of inorganic N taken up from the available soil pool depends on the balance between the different processes of the N cycle (Hogberg 1997). Interestingly, differences between the sexes were also found in the temporal patterns of foliar N content (as noted above); it decreased from April to June in both sexes, while further decrease from June to August occurred in females but not in males. This decrease can be attributed to the increasing allocation to reproduction as the season progressed, and the greater depletion in nitrogen in the tissues of females is to be expected since females not only produce flowers but also carry the burden of fruit maturation (especially later in the season). The overall cost of reproduction in terms of nitrogen is evident at the beginning of the season, when not all individuals were flowering and a comparison between reproductive and non-reproductive individuals could be made. At this time, reproductive individuals (of both sexes) had lower N content in their tissues, suggesting some degree of translocation from vegetative to reproductive structures (Karlsson 1994; Poot *et al.* 1996; Saulnier and Reekie 1995). It is plausible that remobilization of nitrogen from leaves to fruits occurs to support a greater demand of nitrogen in developing fruits and seeds. The internal recycling of nitrogen has been found to affect leaf  $\delta^{15}N$  and the lack of a consistent pattern

indicates that such effect is likely to be species specific (Kolb and Evans 2002).

In conclusion, our study has found differences in physiological traits between males and females of *H. peploides*. Overall, females were more conservative in the use of water than males, and the differences between the sexes in nitrogen use and acquisition strategies depended on time, highlighting the importance of studying sexual dimorphism at different times throughout the season. Males and females seem to occupy similar habitats, and the intersexual differences in physiological traits shown here can be mainly explained as the result of specialization to meet the specific demands associated to reproduction.

## SUPPLEMENTARY MATERIAL

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